The impact of sub-microscopic malaria parasitaemia on rapid diagnosis in north-eastern Tanzania, an area with diverse transmission patterns

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Abstract

Global malaria epidemiology has changed in the last decade with a substantial increase in cases and death being recorded. Over 90% of global cases and deaths occur in Sub-Saharan Africa (SSA). Tanzania accounts for about 4% of all cases and deaths reported in recent years. It is believed that several factors contribute to the resurgence of malaria, parasite resistance to antimalarials and mosquito resistance to insecticides being at the top of the list. The presence of sub-microscopic infections poses a significant challenge to malaria rapid diagnostic tests (mRDT), particularly in low-endemic areas. Our cross-sectional surveys in Handeni and Moshi, Tanzania assessed the effect of low parasite density on mRDT. A significant difference ($P<0.001$) in malaria prevalence by mRDT, light microscopy (LM) and nested polymerase chain reaction (PCR) was found among age groups. In comparison to all other groups, school-age children (5-15 years) had the highest prevalence of malaria. Based on the results of this study, mRDT may miss up to 6% of cases of malaria mainly due to low-density parasitaemia. Routinely used mRDT will likely miss the sub-microscopic parasitemia which will ultimately contribute to the continued spread of malaria and hinder efforts to control and eliminate it.

Keywords: *Malaria transmission, Sub-microscopic, low-density, parasitaemia, rapid diagnostic test*

Introduction

In recent years, there has been little progress in the fight against malaria globally and malaria cases and deaths are on the rise [1]. According to the World malaria report of 2021, malaria cases and deaths increased by about 14 million and 67,000 respectively from 2019 to 2020[1]. The World Health Organization (WHO)-African region contributes to 95% of global cases,

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with six Sub-Saharan countries accounting for about 55% of the cases globally[1]. National malaria surveillance systems in Sub-Saharan Africa (SSA) countries are centered on the detection of symptomatic cases by either mRDTs or light microscopy (LM) [2]. Symptomatic cases fluctuate with transmission season and usually, the peak is just after the rainy season which correlates well with an increase in *Anopheles* mosquito densities[3,4]. Studies suggest that a history of previous malaria exposure can protect some people from severe manifestation of the disease particularly in high transmission areas[5]. There is evidence that naturally-acquired immunity against malaria parasites can inhibit merozoite invasion to the red blood cells (RBCs) and thus suppress the multiplication of the parasites to maintain low-density parasitaemia[6,7].

Individuals with asymptomatic and sub-microscopic infections rarely seek medical treatment and they can act as potential reservoirs for malaria transmission[8–11]. Sub-microscopic infections have been suspected to facilitate human-mosquito transmission in low-endemic areas[12]. Studies suggest that the right gametocyte micro to macro ratio of fewer than 5 gametocytes/µl is enough for transmission to occur [13,14]. This asexual parasite density is below the detection threshold of microscopic analysis.

The low-density parasitaemia will likely contribute to the new transmission circles when mosquito numbers increase in the rainy seasons[9]. Sub microscopic infections pose a serious challenge to parasite detection as a prerequisite for malaria treatment.

The introduction of mRDT in Tanzania more than ten years ago, led to the replacement of LM in most health facilities [15]. Although mRDT has more advantages over LM, it still faces some operational challenges throughout its supply chain[16]. WHO recommends procurement of mRDTs with at least a 75% panel detection score (PDS) at 200 parasites/µl. It is very likely that with the WHO recommended mRDT limit of detection, 25% of infections will be missed and sustain ongoing transmission[10,17]. While microscopy has a detection limit of 50 parasites/µl, which is better than mRDT, nucleic acid detection methods such as PCR are the most sensitive with a detection threshold of approximately 0.2 parasites/µl[18,19].

This study aimed at investigating the impact of sub-microscopic malaria infections on mRDT in areas with diverse malaria transmission endemicity.

Materials and Methods

Description of the study areas

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The study was conducted in Handeni, Tanga region and Moshi in Kilimanjaro region [Fig. 1). Residents from the two areas with the age of 6 months and above were recruited. Handeni is endemic for malaria with a perennial transmission pattern and prevalence of about 28%[20]. The study area has two rainy seasons per year which denotes the peaks of malaria transmission. The long rainy season is from March-June and the short rainy season is from October-November. The area is located 309 meters above sea level. Residents in Handeni engage themselves with small-scale farming and livestock husbandry.

The second study site, Moshi, is located 10 kilometres from Moshi municipality, and 800 meters above sea level, south of Mount Kilimanjaro. Most of the population in the area is engaged in agriculture with irrigated rice and sugarcane cultivation as the main crops. Irrigation activities provide an important breeding site for *Anopheles arabiensis*, the predominant malaria vector in the area. Lower Moshi is a low malaria-endemic area with a prevalence of about 1% in the last 10 years[21].

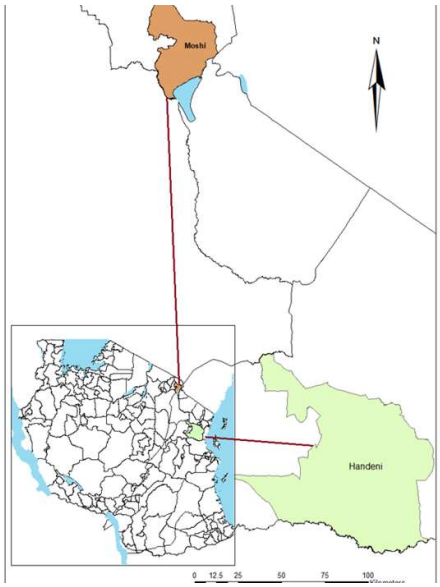

Figure 1: A map of Tanzania showing the study sites (map created using ArcGIS software v10.3)

Participants recruitment and sample collection

A community sensitization campaign was carried out in the study areas, where village residents were invited to designated dispensaries to participate in the study. Potential participants were informed in detail of the study and invited to voluntarily participate in the study by signing an informed consent form.

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Following consenting, a finger prick blood was collected for mRDT, microscopy and for dried blood spots. Additionally, 0.5ml blood sample was drawn by venepuncture for molecular analysis. Blood spots were prepared from 50 µl of blood on Whatman® protein saver cards (Cytiva Plc, USA) followed by drying at room temperature overnight. Dried filter papers were stored in a bag containing silica gel at room temperature.

A blood sample of 0.5 ml was obtained from each participant by venipuncture for molecular analysis.

Sampling in both the study sites was done at the end of the rainy season between April and June 2018.

Malaria rapid diagnosis

Malaria detection on site was done using SD BOLINE pf /pan Ag test kit (Standard Diagnostic INC-Korea) following testing procedures as per manufacturer's instructions. Participants found to be positive were treated using Artemether-lumefantrine (ALu), the recommended first-line antimalarial treatment for uncomplicated malaria in Tanzania.

Microscopic examination

Thick and thin blood smears were prepared using freshly collected blood. The thin smear was fixed using absolute methanol. Both smears were stained for 10 minutes with 10% Giemsa solution, washed with distilled water and dried on a slide warmer (Fischer scientific, USA). Slides were packed in slide boxes and stored at room temperature until reading. Slide reading and recording was done as described in the WHO protocols. [22].

DNA extraction and PCR

The DNA extraction was done following a modified-chelex method [23], where filter paper discs of 8mm diameter were cut from the dried blood spots and placed in a 1.5ml microcentrifuge tube and 1 ml of 0.5% Saponin/Phosphate Buffered Saline (PBS) solution was added to each sample tube, inverted several times and incubated at 4°C overnight to remove haem from the filter paper discs. On the following day, all 0.5% saponin/PBS solution was aspirated and 1.0ml cold PBS solution was added to the filter paper containing tubes followed by vortexing for 15 seconds and incubation at 4°C for 15 minutes after which the PBS solution was aspirated and discarded. 150µl of 6% Chelex solution in DNase/RNase free water was added to each sample. Samples were then heated in a heat block at 100°C for 30 minutes followed by spinning at 12,000rpm for 5 minutes to settle down Chelex. About 100µL of the

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supernatant (DNA-containing solution) was transferred into a new 1.5ml microcentrifuge tube and stored at –20ºC until use.

Plasmodium falciparum was detected using a nested-PCR (PCR) technique targeting 18S ribosomal RNA, as previously described by Snounou *et al*. [24].

Statistical analysis

Statistical analysis was conducted using Stata 16 software (StataCorp LLC, TX, USA). Parasitaemia was log-transformed and presented in a scatter plot. Proportions and frequencies were presented in tables and figures. Wilcoxon rank-sum (Mann – Whitney) test was used to test statistical differences between age groups if $p < 0.05$.

Results

Malaria prevalence by mRDT, LM, and PCR

The study included 998 participants, of which 526 (52.7%) were from Handeni and 472 (47.3%) from Moshi. Majority of the participants were over 15 years of age, constituting 54.1%. Children aged 5-15 years and < 5years comprised 23.5% and 22.4%, respectively.

Malaria prevalence by mRDT, LM and PCR was significantly higher in Handeni ($P< 0.001$) when compared to Moshi. Furthermore, when comparing the individual tests within sites, mRDT detected significantly higher proportion of malaria cases in Handeni site than LM and PCR ($P< 0.001$) different from Moshi site, where PCR detected more malaria cases than RDT and LM ($P< 0.001$). There were also significant differences in malaria prevalence across age groups in Handeni. School-age children (5-15 years) had a higher proportion of malaria cases than the rest of the age groups ($P< 0.001$) in Handeni site. Malaria prevalence in Moshi was too low to perform age-wise analysis **(Table. 1**

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Table 1: **Malaria prevalence by mRDT, LM and PCR across age-groups in Handeni and Moshi sites**

	mRDT				LM				PCR			
N= 998	**Prevalence (%)**	**95% C.I.**		**p-value**	**Prevalence (%)**	**95% C.I.**		**p-value**	**Prevalence (%)**	**95% C.I.**		**p-value**
		Lower	**Upper**			**Lower**	**Upper**			**Lower**	**Upper**	
Sex												
Male	19.9	15.1	24.6	0.723	7.7	4.5	10.9	0.298	12.9	8.9	16.8	0.7816
Female	20.9	17.9	23.9		9.9	7.7	12.1		13.5	11.0	16.1	
Age in years												
<5	25.3	19.6	31.1	<0.001	12.0	7.7	16.3	0.001*	15.7	10.8	20.5	<0.001*
5-15	42.5	36.1	49.0		17.1	12.2	22.0		25.4	19.8	31.1	
>15	9.7	7.2	12.3		5.3	3.4	7.3		7.6	5.4	9.9	
Study site *(N)*												
Handeni (526)	39.6	35.3	43.8	<0.001	16.9	13.7	20.1	<0.001	22.1	18.5	25.6	<0.001
Moshi (472)	0.2	-0.2	0.6		1.3	0.3	2.3		4.0	2.3	5.8	

*Although the overall prevalence of malaria across all age groups was significant, the confidence intervals for age <5 and 5-15 overlap, showing no statistical difference for these two age groups.

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Parasitaemia levels across age groups

Children under five years of age had the highest median parasitaemia (3,162 p/µl) when compared to other age groups. There was a significant difference in parasitaemia between participants with the age below 5 years and those aged >15 years (p-value = 0.0284), with the median parasitaemia of 159 p/µl and 100 p/µl respectively. There was no significant difference in parasitaemia between school-age children and those below five years of age (**Fig 2**).

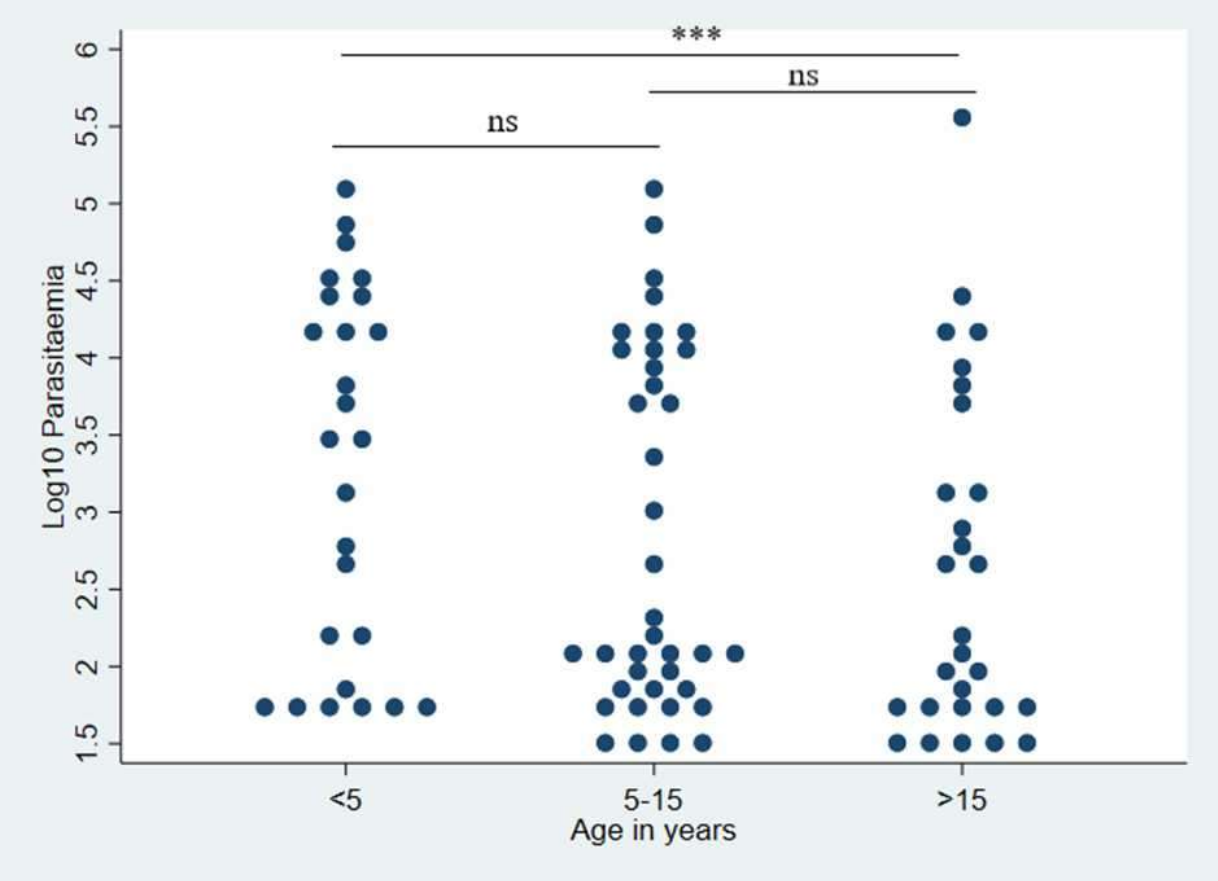

Figure 2. A comparison of parasitaemia across age groups in both Handeni and Moshi. Significance differences are shown as: *** (Significant, P-value = 0.0284), **ns** (not significant)

Sub-microscopic parasitaemia

Of the 204 total positive samples in all study sites, (26) 13% were positive by PCR alone, and almost equally distributed in Handeni and Moshi (12) 46% and (14) 54% respectively. Most of the samples had low parasitaemia, 22 of the 26 (85%) had parasite density below 200 p/µl and only 2 samples had high parasitaemia. More than 58% (15) of sub-microscopic cases were from participants with 15 years and above followed by school-age children (39 %) and children below 5 years of age (3 %) (**Fig. 3**).

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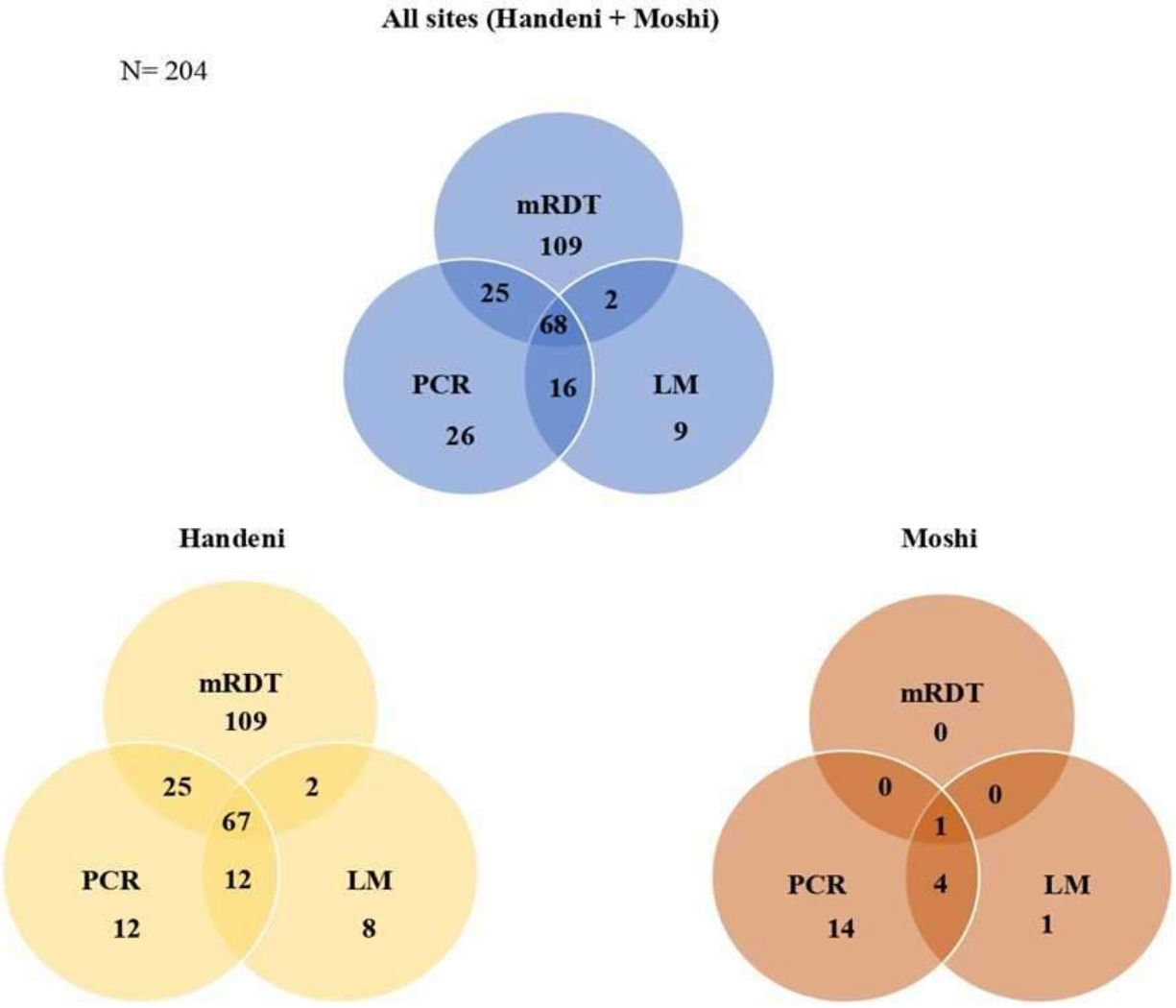

Figure 3. A Venn diagram showing overlaps in malaria detection by different diagnostic tools in the study sites.

Discussion

In this community-based study, we report a considerable difference in malaria prevalence between the study sites. Handeni had a significantly higher prevalence when compared to Moshi. Based on the historical malaria transmission patterns in Handeni, a lowland inhabited by anthropophilic *Anopheles gambiae ss* and *Anopheles funestus* mosquitoes, this difference was expected[25,26]. *Anopheles arabiensis*, which are zoophilic in nature, are abundant in

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Moshi, with a low transmission rate[27].These results are consistent with recent studies that were conducted in the study sites which reported almost similar findings[20,21,28].

Rapid test (mRDT) detected 2 folds more cases in Handeni compared to LM and PCR, suggesting presence of false-positive results. This was different with Moshi site, whereby, PCR detected 20 folds more cases than mRDT. Observation from several studies in high malaria transmission areas estimates up to 5% false positivity due to circulating HRP2 antigen up to five weeks after malaria treatment[29]. Some studies have highlighted mRDT cross-reactivity with immunological agents such as rheumatoid factor (RF) and Human-Anti-Mouse Antibody (HAMA), whereby a 26% false positivity was reported[30,31]. Sub-microscopic parasitaemia is evident in lower endemic areas like Moshi and these will maintain transmission through seasons[12]. False positivity ramifications are far less as compared to false negativity. False-positive individuals will still receive malaria treatment instead of the actual disease they are suffering from. Treating false-positive malaria cases wastes resources and delays the treatment of non-malaria conditions[30].

An age-dependent prevalence analysis showed that school-age children had higher malaria prevalence than the other groups, even though they represented only a quarter of all study participants. Children under the age of five and pregnant women are the main targets of malaria interventions. There is a significant disparity in the delivery of bed nets whereby school-age children are prioritized the least, despite being equally at risk, and this could be the reason for their high malaria prevalence [32,33]. This analysis was not done in Moshi due to a very low malaria prevalence. Several studies have noted an increase in the risk of malaria infection among school-age children over the past decade, and recommendations are made to include this age group as vulnerable as well[32,34,35].

Findings from this study showed a high proportion of sub-microscopic parasitaemia in adults (> 15 years) and children of age between 5 and 15 years. This explains the possibility that Adults and school-age children also experiences repeated malaria exposure and can sustain low density parasitaemia[36]. Asymptomatic sub microscopic parasitaemia was more prominent in adults but recently we have observed a paradigm shift to school-age children making them a potential parasite reservoir[37,38].

A sub-microscopic/low-density infection results from immunological inhibition of parasite multiplication [39–42]. As a result of exposure to parasites over long periods. Adults have

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protective immunity to severe manifestations of the disease[43,44]. Observations from studies in SSA suggest that more frequent exposure to malaria in school-age children may have led to increased malaria parasite-specific immunity and asymptomatic parasite persistence[45,46].

Results from this study showed that mRDT failed to detect 6 cases and 4 cases in every 100 participants in Handeni and Moshi respectively when compared to LM and PCR both combined. Since we have already submitted data on *pfhrp2* deletion, we ruled out the possibility of false negativity due to deletion. High detection threshold of mRDT compared to LM and PCR could also support this finding [47]. This raise concerns if mRDT is the appropriate diagnostic tool in low malaria endemic areas like Moshi. Findings elsewhere in SSA have demonstrated the possibility of mRDT missing malaria cases due to the low limit acceptable detection threshold set by WHO and that could have ramification in elimination campaigns.[48–50].

This cross-sectional study reported a substantial proportion of cases of low parasitaemia (< 200 p/µl) from Handeni and Moshi. There were also two cases with high parasitaemia (>300,000 p/µl) and negative mRDT results. There have been studies showing that these submicroscopic cases tend to be asymptomatic[51,52]. It is thought that high parasitaemia will produce a false negative result in mRDT because of the prozone effect[53,54].

False-negative mRDT is common in low-endemic areas and is believed to have implications for malaria elimination. The evidence of sub-microscopic infections in low malaria-endemic areas suggests that up to 50% of seasonal transmission is attributed to these infections[37]. Studies that aimed at mass testing and treatment, failed to show the reduction of malaria transmission due to the use of mRDT as a diagnostic tool in areas earmarked for pre-elimination [55,56]. Clusters of sub-microscopic infections are stable through low and high transmission seasons. Targeting these clusters can significantly reduce malaria transmission and warrant success in malaria control and elimination.

Conclusion

There is evidence that low-density parasitaemia infections occur in both high and low malaria transmission settings. Although mRDT are easy to use and convenient in low resource settings, they have a higher detection threshold than LM and PCR, making them less viable in low-endemic settings with high sub microscopic malaria prevalence. Sub-microscopic malaria will ultimately facilitate human-mosquito transmission and the effect is more pronounced in low-

endemic areas and that hinders elimination campaigns. A comparative evaluation of the relative benefits of mRDT should be conducted in areas where malaria prevalence is less than 5%.

Author Contributions:

Proposal development, RDK, and RAK; Participants recruitment and sample collection, RDK, and BAS; Laboratory assays, RDK and BAS; Data analysis, FFT and RDK; resources, FWM, DCK; Original draft manuscript preparation, RDK; Manuscript review and editing, All authors; Project supervision, RAK and FWM; funding acquisition, RDK.

All authors have read and agreed to the final version of the manuscript.

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Ethics Review

The study was conducted according to the guidelines of the Declaration of Helsinki and approved by the Institutional Ethics Review Committee) of Kilimanjaro Christian Medical University College (Proposal # 1084, Ethics clearance certificate # 2238 in 2017).

Informed Consent

Informed consent was obtained from all participants involved in the study, by signing the consent form participants also consent to the dissemination of results to conferences and publications.

Data Availability

Data sets developed during this investigation are not publicly available, but can be requested from the corresponding author upon reasonable notice.

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Conflicts of Interest

The authors declare no conflict of interest. Funders did not participate in any aspect of the study design, collection, analysis, interpretation, manuscript writing, or decision-making regarding the publication of the results.

References

1. Organization, W.H. *World malaria report 2021*; World Health Organization: Geneva PP - Geneva, 2021; ISBN 9789240040496 (electronic version).
2. Organization, W.H. *Malaria surveillance, monitoring and evaluation: a reference manual*; World Health Organization: Geneva PP - Geneva, 2018; ISBN 9789241565578.
3. Ouédraogo, A.L.; de Vlas, S.J.; Nébié, I.; Ilboudo-Sanogo, E.; Bousema, J.T.; Ouattara, A.S.; Verhave, J.P.; Cuzin-Ouattara, N.; Sauerwein, R.W. Seasonal patterns of Plasmodium falciparum gametocyte prevalence and density in a rural population of Burkina Faso. *Acta Trop.* **2008**, *105*, 28–34, doi:https://doi.org/10.1016/j.actatropica.2007.09.003.
4. Geiger, C.; Agustar, H.K.; Compaoré, G.; Coulibaly, B.; Sié, A.; Becher, H.; Lanzer, M.; Jänisch, T. Declining malaria parasite prevalence and trends of asymptomatic parasitaemia in a seasonal transmission setting in north-western Burkina Faso between 2000 and 2009–2012. *Malar. J.* **2013**, *12*, 27, doi:10.1186/1475-2875-12-27.
5. Dent, A.E.; Nakajima, R.; Liang, L.; Baum, E.; Moormann, A.M.; Sumba, P.O.; Vulule, J.; Babineau, D.; Randall, A.; Davies, D.H.; et al. Plasmodium falciparum Protein Microarray Antibody Profiles Correlate With Protection From Symptomatic Malaria in Kenya. *J. Infect. Dis.* **2015**, *212*, 1429–1438, doi:10.1093/infdis/jiv224.
6. Niang, M.; Bei, A.K.; Madnani, K.G.; Pelly, S.; Dankwa, S.; Kanjee, U.; Gunalan, K.; Amaladoss, A.; Yeo, K.P.; Bob, N.S.; et al. STEVOR is a plasmodium falciparum erythrocyte binding protein that mediates merozoite invasion and rosetting. *Cell Host Microbe* **2014**, *16*, 81–93, doi:10.1016/j.chom.2014.06.004.
7. Gonzales, S.J.; Reyes, R.A.; Braddom, A.E.; Batugedara, G.; Bol, S.; Bunnik, E.M. Naturally Acquired Humoral Immunity Against Plasmodium falciparum Malaria. *Front. Immunol.* **2020**, *11*, 2809, doi:10.3389/fimmu.2020.594653.
8. Noor, A.M.; Kirui, V.C.; Brooker, S.J.; Snow, R.W. The use of insecticide treated nets by age: implications for universal coverage in Africa. *BMC Public Health* **2009**, *9*, 369, doi:10.1186/1471-2458-9-369.
9. Alves, F.P.; Gil, L.H.S.; Marrelli, M.T.; Ribolla, P.E.M.; Camargo, E.P.; Da Silva, L.H.P. Asymptomatic Carriers of Plasmodium spp. as Infection Source for Malaria

Vector Mosquitoes in the Brazilian Amazon . *J. Med. Entomol.* **2005**, *42*, 777–779, doi:10.1093/jmedent/42.5.777.

10. Zaw, M.T.; Thant, M.; Hlaing, T.M.; Aung, N.Z.; Thu, M.; Phumchuea, K.; Phusri, K.; Saeseu, T.; Yorsaeng, R.; Nguitragool, W.; et al. Asymptomatic and sub-microscopic malaria infection in Kayah State, eastern Myanmar. *Malar. J.* **2017**, *16*, 138, doi:10.1186/s12936-017-1789-9.

11. Zoghi, S.; Mehrizi, A.A.; Raeisi, A.; Haghdoost, A.A.; Turki, H.; Safari, R.; Kahanali, A.A.; Zakeri, S. Survey for asymptomatic malaria cases in low transmission settings of Iran under elimination programme. *Malar. J.* **2012**, *11*, 126, doi:10.1186/1475-2875-11-126.

12. Shekalaghe, S.A.; Bousema, J.T.; Kunei, K.K.; Lushino, P.; Masokoto, A.; Wolters, L.R.; Mwakalinga, S.; Mosha, F.W.; Sauerwein, R.W.; Drakeley, C.J. Submicroscopic Plasmodium falciparum gametocyte carriage is common in an area of low and seasonal transmission in Tanzania. *Trop. Med. Int. Health* **2007**, *12*, 547–553, doi:10.1111/j.1365-3156.2007.01821.x.

13. Zhou, Z.; Mitchell, R.M.; Kariuki, S.; Odero, C.; Otieno, P.; Otieno, K.; Onyona, P.; Were, V.; Wiegand, R.E.; Gimnig, J.E.; et al. Assessment of submicroscopic infections and gametocyte carriage of Plasmodium falciparum during peak malaria transmission season in a community-based cross-sectional survey in western Kenya, 2012. *Malar. J.* **2016**, *15*, 421, doi:10.1186/s12936-016-1482-4.

14. Schneider, P.; Bousema, J.T.; Gouagna, L.C.; Otieno, S.; van de Vegte-Bolmer, M.; Omar, S.A.; Sauerwein, R.W. Submicroscopic Plasmodium falciparum gametocyte densities frequently result in mosquito infection. *Am. J. Trop. Med. Hyg.* **2007**, *76*, 470–474.

15. Masanja, I.M.; Selemani, M.; Amuri, B.; Kajungu, D.; Khatib, R.; Kachur, S.P.; Skarbinski, J. Increased use of malaria rapid diagnostic tests improves targeting of anti-malarial treatment in rural Tanzania: implications for nationwide rollout of malaria rapid diagnostic tests. *Malar. J.* **2012**, *11*, 221, doi:10.1186/1475-2875-11-221.

16. Albertini, A.; Lee, E.; Coulibaly, S.O.; Sleshi, M.; Faye, B.; Mationg, M.L.; Ouedraogo, K.; Tsadik, A.G.; Feleke, S.M.; Diallo, I.; et al. Malaria rapid diagnostic test transport and storage conditions in Burkina Faso, Senegal, Ethiopia and the Philippines. *Malar. J.* **2012**, *11*, 406, doi:10.1186/1475-2875-11-406.

17. WHO Malaria rapid diagnostic test performance. Results of WHO product testing of malaria RDTs: round 8 2018, 172.

18. Milne, L.M.; Kyi, M.S.; Chiodini, P.L.; Warhurst, D.C. Accuracy of routine laboratory diagnosis of malaria in the United Kingdom. *J. Clin. Pathol.* **1994**, *47*, 740–742, doi:10.1136/jcp.47.8.740.

19. Li, P.; Zhao, Z.; Wang, Y.; Xing, H.; Parker, D.M.; Yang, Z.; Baum, E.; Li, W.; Sattabongkot, J.; Sirichaisinthop, J.; et al. Nested PCR detection of malaria directly using blood filter paper samples from epidemiological surveys. *Malar. J.* **2014**, *13*, 175, doi:10.1186/1475-2875-13-175.

20. Kaaya, R.D.; Kajeguka, D.C.; Matowo, J.J.; Ndaro, A.J.; Mosha, F.W.; Chilongola, J.O.; Kavishe, R.A. Predictive markers of transmission in areas with different malaria endemicity in north-eastern Tanzania based on seroprevalence of antibodies against

Plasmodium falciparum. *BMC Res. Notes* **2021**, *14*, 404, doi:10.1186/s13104-021-05818-y.

21. Kassam, N.A.; Kaaya, R.D.; Damian, D.J.; Schmiegelow, C.; Kavishe, R.A.; Alifrangis, M.; Wang, C.W. Ten years of monitoring malaria trend and factors associated with malaria test positivity rates in Lower Moshi. *Malar. J.* **2021**, *20*, 193, doi:10.1186/s12936-021-03730-1.

22. Organization, W.H. Basic malaria microscopy 2010.

23. Miguel, R.B.; Coura, J.R.; Samudio, F.; Suárez-Mutis, M.C. Evaluation of three different DNA extraction methods from blood samples collected in dried filter paper in Plasmodium subpatent infections from the Amazon region in Brazil. *Rev. Inst. Med. Trop. Sao Paulo* **2013**, *55*, doi:10.1590/S0036-46652013000300012.

24. Snounou, G.; Singh, B. Nested PCR analysis of Plasmodium parasites. *Methods Mol. Med.* **2002**, *72*, 189–203, doi:10.1385/1-59259-271-6:189.

25. Emidi, B.; Kisinza, W.N.; Kaaya, R.D.; Malima, R.; Mosha, F.W. Insecticide susceptibility status of human biting mosquitoes in Muheza, Tanzania. *Tanzan. J. Health Res.* **2017**, *19*.

26. Kaindoa, E.W.; Matowo, N.S.; Ngowo, H.S.; Mkandawile, G.; Mmbando, A.; Finda, M.; Okumu, F.O. Interventions that effectively target Anopheles funestus mosquitoes could significantly improve control of persistent malaria transmission in south-eastern Tanzania. *PLoS One* **2017**, *12*, e0177807–e0177807, doi:10.1371/journal.pone.0177807.

27. Kitau, J.; Oxborough, R.M.; Tungu, P.K.; Matowo, J.; Malima, R.C.; Magesa, S.M.; Bruce, J.; Mosha, F.W.; Rowland, M.W. Species shifts in the Anopheles gambiae complex: do LLINs successfully control Anopheles arabiensis? *PLoS One* **2012**, *7*, e31481, doi:10.1371/journal.pone.0031481.

28. Hayuma, P.M.; Wang, C.W.; Liheluka, E.; Baraka, V.; Madebe, R.A.; Minja, D.T.R.; Misinzo, G.; Alifrangis, M.; Lusingu, J.P.A. Prevalence of asymptomatic malaria, submicroscopic parasitaemia and anaemia in Korogwe District, north-eastern Tanzania. *Malar. J.* **2021**, *20*, 424, doi:10.1186/s12936-021-03952-3.

29. Dalrymple, U.; Arambepola, R.; Gething, P.W.; Cameron, E. How long do rapid diagnostic tests remain positive after anti-malarial treatment? *Malar. J.* **2018**, *17*, 228, doi:10.1186/s12936-018-2371-9.

30. Gatton, M.L.; Ciketic, S.; Barnwell, J.W.; Cheng, Q.; Chiodini, P.L.; Incardona, S.; Bell, D.; Cunningham, J.; González, I.J. An assessment of false positive rates for malaria rapid diagnostic tests caused by non-Plasmodium infectious agents and immunological factors. *PLoS One* **2018**, *13*, e0197395, doi:10.1371/journal.pone.0197395.

31. Iqbal, J.; Sher, A.; Rab, A. Plasmodium falciparum histidine-rich protein 2-based immunocapture diagnostic assay for malaria: cross-reactivity with rheumatoid factors. *J. Clin. Microbiol.* **2000**, *38*, 1184–1186, doi:10.1128/JCM.38.3.1184-1186.2000.

32. Cohee, L.M.; Nankabirwa, J.I.; Greenwood, B.; Djimde, A.; Mathanga, D.P. Time for malaria control in school-age children. *Lancet Child Adolesc. Heal.* **2021**, *5*, 537–538, doi:10.1016/S2352-4642(21)00158-9.

33. Olapeju, B.; Choiriyyah, I.; Lynch, M.; Acosta, A.; Blaufuss, S.; Filemyr, E.; Harig, H.;

Monroe, A.; Selby, R.A.; Kilian, A.; et al. Age and gender trends in insecticide-treated net use in sub-Saharan Africa: a multi-country analysis. *Malar. J.* **2018**, *17*, 423, doi:10.1186/s12936-018-2575-z.

34. Chacky, F.; Runge, M.; Rumisha, S.F.; Machafuko, P.; Chaki, P.; Massaga, J.J.; Mohamed, A.; Pothin, E.; Molteni, F.; Snow, R.W.; et al. Nationwide school malaria parasitaemia survey in public primary schools, the United Republic of Tanzania. *Malar. J.* **2018**, *17*, 452, doi:10.1186/s12936-018-2601-1.

35. Cohee, L.M.; Opondo, C.; Clarke, S.E.; Halliday, K.E.; Cano, J.; Shipper, A.G.; Barger-Kamate, B.; Djimde, A.; Diarra, S.; Dokras, A.; et al. Preventive malaria treatment among school-aged children in sub-Saharan Africa: a systematic review and meta-analyses. *Lancet Glob. Heal.* **2020**, *8*, e1499–e1511, doi:10.1016/S2214-109X(20)30325-9.

36. Mathanga, D.P.; Halliday, K.E.; Jawati, M.; Verney, A.; Bauleni, A.; Sande, J.; Ali, D.; Jones, R.; Witek-McManus, S.; Roschnik, N.; et al. The High Burden of Malaria in Primary School Children in Southern Malawi. *Am. Soc. Trop. Med. Hyg.* **2015**, *93*, 779–789, doi:10.4269/ajtmh.14-0618.

37. Okell, L.C.; Bousema, T.; Griffin, J.T.; Ouédraogo, A.L.; Ghani, A.C.; Drakeley, C.J. Factors determining the occurrence of submicroscopic malaria infections and their relevance for control. *Nat. Commun.* **2012**, *3*, 1237, doi:10.1038/ncomms2241.

38. Walldorf, J.A.; Cohee, L.M.; Coalson, J.E.; Bauleni, A.; Nkanaunena, K.; Kapito-Tembo, A.; Seydel, K.B.; Ali, D.; Mathanga, D.; Taylor, T.E.; et al. School-Age Children Are a Reservoir of Malaria Infection in Malawi. *PLoS One* **2015**, *10*, e0134061–e0134061, doi:10.1371/journal.pone.0134061.

39. Yazdani, S.S.; Mukherjee, P.; Chauhan, V.S.; Chitnis, C.E. Immune responses to asexual blood-stages of malaria parasites. *Curr. Mol. Med.* **2006**, *6*, 187–203, doi:10.2174/156652406776055212.

40. Boyd, M.F.; Stratman-Thomas, W.K.; Kitchen, S.F. On Acquired Immunity to Plasmodium Falciparum1. *Am. J. Trop. Med. Hyg.* **1936**, *1*, 139–145.

41. Cabrera, E.J.; Barr, M.L.; Silverman, P.H. Long-term studies on rhesus monkeys (Macaca mulatta) immunized against Plasmodium knowlesi. *Infect. Immun.* **1977**, *15*, 461–465, doi:10.1128/iai.15.2.461-465.1977.

42. Wilson, R.J.; Phillips, R.S. Method to test inhibitory antibodies in human sera to wild populations of Plasmodium falciparum. *Nature* **1976**, *263*, 132–134, doi:10.1038/263132a0.

43. Griffin, J.T.; Déirdre Hollingsworth, T.; Reyburn, H.; Drakeley, C.J.; Riley, E.M.; Ghani, A.C. Gradual acquisition of immunity to severe malaria with increasing exposure. *Proc. R. Soc. B Biol. Sci.* **2015**, *282*, doi:10.1098/rspb.2014.2657.

44. van den Hoogen, L.L.; Walk, J.; Oulton, T.; Reuling, I.J.; Reiling, L.; Beeson, J.G.; Coppel, R.L.; Singh, S.K.; Draper, S.J.; Bousema, T.; et al. Antibody Responses to Antigenic Targets of Recent Exposure Are Associated With Low-Density Parasitemia in Controlled Human Plasmodium falciparum Infections . *Front. Microbiol.* 2019, *9*.

45. Mensah, B.A.; Myers-Hansen, J.L.; Obeng Amoako, E.; Opoku, M.; Abuaku, B.K.; Ghansah, A. Prevalence and risk factors associated with asymptomatic malaria among

school children: repeated cross-sectional surveys of school children in two ecological zones in Ghana. *BMC Public Health* **2021**, *21*, 1697, doi:10.1186/s12889-021-11714-8.

46. Worku, L.; Damtie, D.; Endris, M.; Getie, S.; Aemero, M. Asymptomatic Malaria and Associated Risk Factors among School Children in Sanja Town, Northwest Ethiopia. *Int. Sch. Res. Not.* **2014**, *2014*, 303269, doi:10.1155/2014/303269.

47. Berzosa, P.; de Lucio, A.; Romay-Barja, M.; Herrador, Z.; González, V.; García, L.; Fernández-Martínez, A.; Santana-Morales, M.; Ncogo, P.; Valladares, B.; et al. Comparison of three diagnostic methods (microscopy, RDT, and PCR) for the detection of malaria parasites in representative samples from Equatorial Guinea. *Malar. J.* **2018**, *17*, 333, doi:10.1186/s12936-018-2481-4.

48. Acquah, F.K.; Donu, D.; Obboh, E.K.; Bredu, D.; Mawuli, B.; Amponsah, J.A.; Quartey, J.; Amoah, L.E. Diagnostic performance of an ultrasensitive HRP2-based malaria rapid diagnostic test kit used in surveys of afebrile people living in Southern Ghana. *Malar. J.* **2021**, *20*, 125, doi:10.1186/s12936-021-03665-7.

49. Poti, K.E.; Sullivan, D.J.; Dondorp, A.M.; Woodrow, C.J. HRP2: Transforming Malaria Diagnosis, but with Caveats. *Trends Parasitol.* **2020**, *36*, 112–126, doi:10.1016/j.pt.2019.12.004.

50. Oladosu, O.; Adedokun Victoria, A.; Adeniyi Akinkunle, V.; Oyibo Wellington, A. Performance Evaluation of Malaria HRP-2 Rapid Diagnostic Test among Febrile Patients with Malaria in Iwo, Osun State, Nigeria. *Int. J. Trop. Dis.* **2021**, *4*, doi:10.23937/2643-461x/1710046.

51. Baum, E.; Sattabongkot, J.; Sirichaisinthop, J.; Kiattibutr, K.; Jain, A.; Taghavian, O.; Lee, M.C.; Huw Davies, D.; Cui, L.; Felgner, P.L.; et al. Common asymptomatic and submicroscopic malaria infections in Western Thailand revealed in longitudinal molecular and serological studies: A challenge to malaria elimination. *Malar. J.* **2016**, *15*, 1–15, doi:10.1186/s12936-016-1393-4.

52. van Eijk, A.M.; Sutton, P.L.; Ramanathapuram, L.; Sullivan, S.A.; Kanagaraj, D.; Priya, G.S.L.; Ravishankaran, S.; Asokan, A.; Sangeetha, V.; Rao, P.N.; et al. The burden of submicroscopic and asymptomatic malaria in India revealed from epidemiology studies at three varied transmission sites in India. *Sci. Rep.* **2019**, *9*, 17095, doi:10.1038/s41598-019-53386-w.

53. Santos, L.; Pereira, N.R.; Andrade, P.; Dias, P.F.; Alves, C.L.; Abreu, C.; Serrão, R.; Ribeiro, M.; Sarmento, A. Prozone-like phenomenon in travellers with fatal malaria: Report of two cases. *J. Infect. Dev. Ctries.* **2015**, *9*, 321–324, doi:10.3855/jidc.5454.

54. Luchavez, J.; Baker, J.; Alcantara, S.; Belizario, V.; Cheng, Q.; McCarthy, J.S.; Bell, D. Laboratory demonstration of a prozone-like effect in HRP2-detecting malaria rapid diagnostic tests: implications for clinical management. *Malar. J.* **2011**, *10*, 286, doi:10.1186/1475-2875-10-286.

55. Sutanto, I.; Kosasih, A.; Elyazar, I.R.F.; Simanjuntak, D.R.; Larasati, T.A.; Dahlan, M.S.; Wahid, I.; Mueller, I.; Koepfli, C.; Kusriastuti, R.; et al. Negligible Impact of Mass Screening and Treatment on Mesoendemic Malaria Transmission at West Timor in Eastern Indonesia: A Cluster-Randomized Trial. *Clin. Infect. Dis.* **2018**, *67*, 1364–1372, doi:10.1093/cid/ciy231.

56. Cook, J.; Xu, W.; Msellem, M.; Vonk, M.; Bergström, B.; Gosling, R.; Al-Mafazy, A.-

 doi:10.20944/preprints202202.0086.v1

W.; McElroy, P.; Molteni, F.; Abass, A.K.; et al. Mass Screening and Treatment on the Basis of Results of a Plasmodium falciparum-Specific Rapid Diagnostic Test Did Not Reduce Malaria Incidence in Zanzibar. *J. Infect. Dis.* **2015**, *211*, 1476–1483, doi:10.1093/infdis/jiu655.